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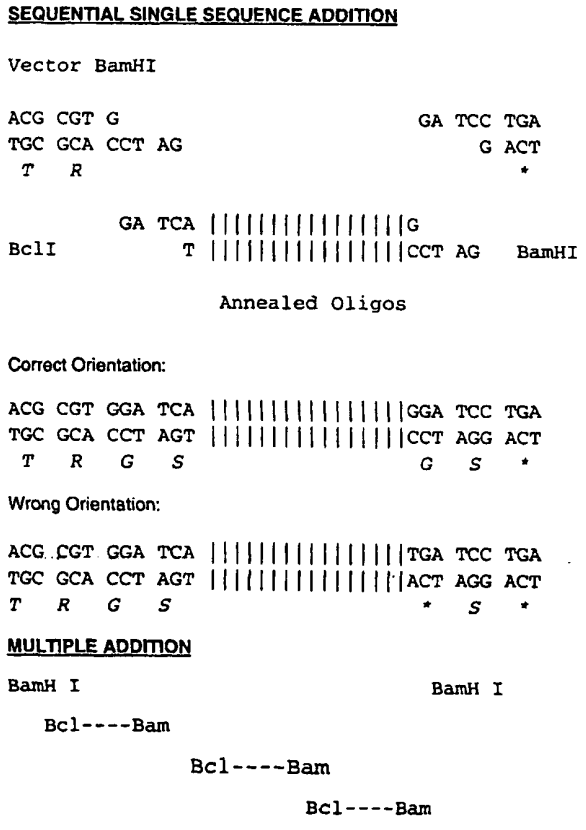
(54) Title: COMBINATORIAL METHOD FOR PRODUCING NUCLEIC ACIDS

(57) Abstract

A method of generating novel nucleic acid molecules, by applying a combinatorial approach to the assembly of blocks of nucleic acid sequence, is described. By using restriction endonucleases having different recognition sites but which produce compatible cleavage products, a library of DNA molecules, of varying length and sequence, may be generated in a desired orientation.



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COMBINATORIAL METHOD FOR PRODUCING NUCLEIC ACIDS

The present invention relates to a method for the generation of novel nucleic acid molecules and proteins, and to nucleic acids and proteins produced by such a method. The invention allows the generation *in vitro* of new biological products formed by applying a combinatorial approach to blocks of nucleic acid sequence.

Advances in recombinant DNA technology over the last decade or so have meant that it has become possible to construct synthetic genes and, consequently, synthetic proteins. Molecules may now be rationally designed and produced with the aim of improving their efficacy over that of a sequence that occurs naturally.

Various techniques now exist for the generation of libraries of proteins, most using methods that allow the random combination of a number of peptides to produce a library of variants. Molecules having the desired characteristics can be isolated through selection regimes that select for a desired phenotype, such as a particular biochemical or biological activity.

Phage display provides one example of a technology that has been highly successful in allowing for the selection of a displayed protein (for reviews see Clackson and Wells, 1994, Hoogenboom HR, 1997 and Lowman HB, 1997). Additionally, combinatorial chemistry can be used to generate peptides of random sequence (Lom KS, 1997).

Existing methods for the generation of libraries of peptides or proteins are limited by pre-design requirements, the number of molecules in the library and the small size of the products obtained. The present invention advantageously provides a method that facilitates the generation of new genes and proteins of unlimited size, assembled in either a predetermined and/or random order, and also allows their subsequent analysis. This method utilises compatible restriction enzymes and ligation to build DNA molecules from smaller, naturally occurring and/or synthetic DNA in a desired orientation.

Thus according to one aspect of the present invention there is provided a method of generating a library of DNA molecules of varying length and sequence in a desired orientation comprising the steps of:

a) providing a mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which are compatible to each other and correspond to the cleavage products of different but compatible restriction enzymes; and

b) allowing ligation to take place, wherein ligation of said double-stranded DNA molecules in desired orientations generates molecules that are not cut by either of said restriction enzymes whereas ligation in undesired orientations generates molecules that retain at one or more ligation points a restriction site that is recognised by one of said restriction enzymes; and

c) cutting the ligated DNA molecules with one or both of said restriction enzymes such that only molecules that are ligated in undesired orientations are cut, leaving a library of DNA molecules of varying length and sequence in a desired orientation.

Where desired the mixture of double-stranded DNA molecules in step a) may also be ligated to a cut vector. Advantageously this allows for the subsequent analysis and utilisation of the assembled DNA molecules. Thus according to a further aspect of the invention, there is provided a method of generating a library of DNA molecules of varying length and sequence in a desired orientation in a vector comprising the steps of:

a1) cutting a double-stranded DNA vector molecule with a first restriction enzyme.

a2) adding to the cut vector molecule a mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which correspond to the cleavage products of different but compatible restriction enzymes, one of said restriction enzymes being said first restriction enzyme,

b) allowing ligation to take place,

c) cutting the ligated DNA molecules with at least said first restriction enzyme such that molecules that are ligated in an incorrect orientation in the vector are cut out of the vector, and optionally

d) repeating steps (a2) to (c) to leave a library of DNA molecules of varying length and sequence in a desired orientation in a vector.

For ease of description of the invention, each component of the mixture of double-stranded DNA molecules initially present at the start of the method will be referred to hereinafter as a "sequence block" (SB) unless stated otherwise. Each SB will in general be naturally occurring or synthetic DNA. The size of each SB is advantageously not crucial and may be varied widely as desired, for example from a few bases up to and beyond 10Kb in length. Each end of a SB comprises half a restriction enzyme site and may be blunt-ended or preferably single-stranded thereby forming a cohesive end. Each half-site of each SB is compatible with the other. Particular sites include those equivalent to the half-sites obtainable by cutting with the restriction enzymes described hereinafter. Additionally, the SB may contain a recognition site for a restriction enzyme, which is preferably distinct from any recognition site capable of being formed by the half-sites of the SB.

Where desired the SB may define a specific motif. The motif may be biologically functional at the protein or at the nucleic acid level. In the latter case the motif may represent, for example, a promoter element, a binding motif for a regulator or inhibitor protein, a response element, an enhancer element, a nuclease site, a hairpin motif or a spacer or linker domain that is required for the correct assembly of the double-stranded nucleic acid molecule.

In the case of an SB encoding a motif with a biological function as part of a protein, the motif may be a binding domain, especially for example a SH2 or SH3 domain, and a SH2 or SH3 binding domain, a dimerisation domain, a signalling sequence (such as an immunoglobulin tyrosine based activation motif; ITAM), a recognition site for an enzyme, an immunoglobulin domain or fragment thereof, an epitope, a transmembrane domain, a catalytic domain, a regulatory domain, an α helical motif or other structural or functional domain. Particular examples of such domains will be readily clear to the man of skill in the art.

Each SB in the starting mixture may be generated by any convenient method, for example by PCR cloning from naturally occurring complementary DNA sources and subsequent cutting with appropriate restriction enzymes (see for example those described below). This allows the selection of any naturally-occurring sequence module of interest, of almost any length. More usually, the SBs may be generated by

annealing two synthetic strands of nucleic acid (oligonucleotides) so that the 5' end and the 3' end form two appropriate overhangs. Combinations of these two approaches may be applied.

Restriction enzymes for use in the methods according to the invention recognise symmetric double-stranded DNA sequences and cleave within the sequences leaving a 3'-hydroxyl on one side of the cut and a 5' phosphate on the other. Depending on the type of restriction enzyme, a fragment with either a cohesive end (having a 5' or 3' single-stranded overhang) or a blunt-ended end (no single-stranded overhang) is produced. Cohesive DNA fragments can be ligated to other DNA fragments if their single-stranded overhangs are compatible. Depending on the sequence of the cleavage product of each fragment that is ligated together, either the original recognition sequence, a new recognition sequence or even no recognition sequence may be formed on ligation. Different restriction enzymes that produce compatible overhangs, which may be ligated together such that they may or may not produce a recleavable ligation product, are termed "compatible restriction enzymes". When overhangs that have been generated by the same restriction enzyme religate together, they reform a recognition site for that enzyme.

In the method of the invention, compatible restriction enzymes that have a 6 base pair or longer recognition sequence are preferred, since such enzymes cut DNA infrequently. Assuming a 50% G-C content of the DNA, a restriction enzyme with a 6 base pair recognition sequence will cleave, on average, every 4^6 (4096) base pairs in a given DNA sequence. Restriction enzymes that produce cohesive ends are preferred since they ligate much more efficiently.

One example of two compatible restriction enzymes that form cohesive ends on digestion is the *Bam*HI/*Bcl*I pair. These enzymes produce overhangs that may be ligated to each other, but which form a ligation product that is not cleavable by either enzyme. In fact, the ligation product may be cleaved by the unrelated enzymes *Alw*I and *Dpn*I.

Preferred restriction enzymes that form cohesive ends are: *Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Bst*EI, *Bst*BI, *Bst*YI, *Eco*RI, *Mlu*I, *Nar*I, *Nhe*I, *Not*I, *Pst*I, *Pvu*I, *Sac*I, *Sal*I, *Spe*I, *Sty*I, *Xba*I, *Xho*I and *Xma*I. Most preferably, the compatible set *Bgl*II, *Bam*HI and *Bcl*I is

used. Preferred enzymes that form blunt ends are *Eco*RV, *Fsp*I, *Nae*I, *Nru*I, *Pvu*II, *Sma*I, *Sna*BI and *Stu*I.

It is envisaged that more than one pair of restriction endonucleases that are different but produce compatible cleavage products, may be employed in the method of the invention.

The recognition sites of these enzymes, the sites with which they are compatible, and the cleavable products are listed in catalogues of most restriction enzyme suppliers, for example the New England Biolabs catalogue.

In the present invention, compatible restriction enzymes are selected on the basis of the amino acid sequence that is desired, the necessity to maintain the reading frame of the protein sequence, and the rarity with which the enzymes cut the chosen DNA sequence. Also relevant is whether during subsequent steps of the process, it will be desirable to recleave at the ligation point.

Where in a method according to the invention the SBs are ligated to a cut vector, the latter may be any double-stranded DNA vector which has been cut with a restriction enzyme, for example, selected from those described above. The vector may contain such features necessary to enable the vector to be grown, maintained and selected in a host cell, including prokaryotic, yeast and higher eukaryotic e.g. mammalian cells.

The vector may be an expression vector and, in this event, may comprise an appropriately-positioned promoter, polyadenylation signal and transcription termination sequence, as well as features to allow expression of a protein encoded by a combination of SBs, such as a ribosome binding site and signal sequences. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if so desired. Expression constructs are normally maintained in a replicon, such as an episomal element (e.g. plasmid) capable of stable maintenance in a host, such as mammalian cells or bacteria. For further details, see *Biological Regulation and Development: Gene Expression* (ed. R.F. Goldberger) and *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.* (1989)).

Suitable vectors for use in the invention are widely available, for example from commercial suppliers such as Clontech Laboratories Inc., Palo Alto, USA and Stratagene, La Jolla, USA, and may be modified as desired using conventional techniques.

Particularly suitable vectors for use in processes according to the invention include viral vectors such as retroviral and adenoviral vectors in mammalian cells.

In general, a desirable feature of the vector is that, once the SBs have been assembled into the vector and analysed, the SB combination(s) of choice may be transferred from the cloning vector and inserted into alternative vectors that allow the function or structure of the assembled SBs to be assessed. In most instances, this will be achieved by utilising a unique restriction site that is recognised by an enzyme that does not cut at any site in the sequence of the assembled SBs.

A vector of choice is a cloning cassette system derived from pBluescript SK+ (Stratagene). This vector is a modification of the cassette system described in International Patent Specification WO97/23613, the contents of which are incorporated herein in their entirety.

Where desired, the process according to the invention may be adapted to use a solid phase. Thus, according to a further aspect of the invention there is provided a method of generating a library of DNA molecules of varying length and sequence in a desired orientation on a solid phase comprising the steps of:

a1) providing a solid phase to which is attached a first double-stranded DNA molecule which has an end corresponding to the cleavage product of a first restriction enzyme;

a2) adding to said solid phase a mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which correspond to the cleavage product of a different but compatible restriction enzyme, one of said restriction enzymes being said first restriction enzyme;

b) allowing ligation to take place, wherein ligation of said double-stranded DNA molecules in a correct orientation generates a molecule that is not cut by either of said

restriction enzymes, whereas ligation in an incorrect orientation retains at one or more ligation points a restriction site that is recognised by one of said restriction enzymes; and

c) cutting the ligated DNA molecules with one or both of said restriction enzymes such that only molecules that are ligated in an incorrect orientation are cut.

The solid phase may comprise any solid matrix to which a DNA molecule may be attached, for example a synthetic bead, column, or any other solid surface. Suitable methods of attachment of DNA molecules to a solid phase are known in the art, such as, for example by biotin capture (Sterky F. *et al* 1998, Journal of Biotechnology 60, 119-125) and by digoxigenin/anti-digoxigenin interaction (Ioannon, P. and Christopoulos, T. 1998, Analytical Chemistry 70, 698-702).

The methods according to the invention employ standard DNA ligation reactions and restriction enzyme digestion to generate a DNA library. Such techniques are well-known and routinely practised, and are described for example in laboratory manuals such as "Molecular Cloning" [Maniatis *et al.*, Cold Spring Harbour Laboratory, New York, 1989]. Suitable ligases, e.g. T4 DNA ligase, and necessary cofactors such as ATP, are commercially available and may be used according to the manufacturer's instructions. Similarly, restriction enzymes, such as those described above are commercially available and may be used as instructed by the supplier.

The ligation step in each method may be controlled by manipulation of SB concentration, incubation time and/or temperature to determine the degree of ligation. For maximum efficiency the ligation conditions, such as SB concentration, may need to be determined empirically, for example by titration, for any particular application.

Where desired ligated SBs may be purified from other components of the ligation reaction, such as enzymes and ATP, using standard separation procedures, for example, by gel exclusion and other size fractionation techniques.

Of course, as will be apparent to the skilled artisan, any of the nucleic acids generated using the methods of the present invention may be mutagenised in accordance with standard techniques as described, for example, in *Molecular Cloning: a Laboratory*

Manual: 2nd edition, (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press) or in *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993). A particularly suitable method for introducing mutations within individual SBs is described below. This method allows the introduction of random mutations at a specific site (or sites) within a SB.

This is achieved through the use of substantially complementary oligonucleotides, one or both of which is degenerate. These oligonucleotides are annealed, ligated into a suitable vector and then transformed into a suitable host. Mutations may thus be introduced at the sites of degeneracy; the precise mutation introduced will depend on the degree of degeneracy at a particular nucleotide position and the operation of the mismatch repair system of the host organism. This will repair any nucleotides that are mismatched between the substantially complementary oligonucleotide pairs during DNA replication, as the cells grow and divide.

Thus, the method of the invention may employ at least one mutant double-stranded DNA molecule, which has been generated by annealing substantially complementary oligonucleotides as described above, the sequences of which are based on a parent nucleic acid, to form a plurality of double-stranded DNA molecules, ligating said plurality of double-stranded DNA molecules with vector, transforming the modified vector molecules into a host cell, culturing said transformed cell under conditions suitable for growth and cell division and isolating mutated DNA from the host cell.

The oligonucleotides are preferably based on a parent nucleic acid. Such parent nucleic acid may encode a polypeptide (this aspect of the invention, where an SB encodes a string of amino acids is described below in more detail).

Mutations may be introduced by annealing complementary oligonucleotides, only one of which is degenerate. Alternatively they may be introduced by annealing complementary oligonucleotides, both of which are degenerate, either at corresponding, or at different, nucleotide positions.

More than one mutation may be introduced into each SB. However, it will be appreciated that there will be a maximum number of mutations that can be introduced at any one time. This number will depend on the length of the oligonucleotide and other factors that govern

efficient annealing. However, we have found that the method works satisfactorily when an oligonucleotide exhibits degeneracy at a ratio equal to, or less than 1 degenerate nucleotide in every 5 nucleotides. This ratio is calculated as an average over the entire length of the oligonucleotide, and is meant to be used only as a guide when designing a degenerate oligonucleotide, as it may be possible to increase the amount of degeneracy in certain circumstances.

In some instances, it may be desirable to cluster degeneracy in, for example, groups of three adjacent nucleotides. Clustering degeneracy in such groups of three is particularly useful if one wishes to effect a change in the amino acid sequence that a particular SB encodes. Thus, an individual codon may be altered by making 1 out of 3, 2 out of 3 or all 3 adjacent nucleotides degenerate. It will be clear to the skilled man that whilst the number of nucleotides in a degenerate cluster may exceed three, the mismatch repair system of the host organism, into which the annealed oligonucleotides will be introduced, will only tolerate and repair a maximum number of unpaired nucleotides before invoking other repair mechanisms, e.g. excision of the unpaired region of DNA.

Whilst this method of mutagenesis allows one to target precisely which nucleotide(s) is mutated, the nature of the mutation will be random due to the degeneracy of the oligonucleotide at the site of mutation. It may be desirable to bias this mutation away from (or towards) a particular nucleotide base, for example, to reduce the probability of introducing a stop codon. This can be achieved by limiting the degree of degeneracy created at a given position within the oligonucleotide. One example of this limited degeneracy would be where only C, G or T (and not A) are present at a particular position. It will be clear to the skilled reader that any desired bias may be introduced in this manner. Alternatively, if the degeneracy of the oligonucleotide at a given position is maximal (i.e. all four nucleotide bases or inosine are present at the desired site of mutation), the mutation will be random.

The conditions that are required for annealing degenerate oligonucleotides will, in general, be similar to those described herein in the examples. However, it will be appreciated by the skilled reader that these conditions may vary and may be dependable on factors such as length of oligonucleotide, the percentage of nucleotides that are degenerate, and the percentage GC content. Annealed oligonucleotides will then be ligated with any suitable

vector and transformed (for example, by calcium chloride transformation, electroporation or any other method that is well known in the art) into a suitable host organism, preferably bacteria or yeast. The host organism will then be cultured under conditions suitable for cell growth and maintenance of the vector, examples of which are readily available in the art, see for example, Sambrook *et al.*, (*Molecular cloning: a laboratory manual.* 2nd edition. Cold Spring Harbour Press, N.Y) and Glover (*DNA cloning: a practical approach, Volume II: Expression systems.* IRL press).

The methods of the invention may be repeated in iterative steps, for example, to incrementally increase in size and complexity the DNA molecules generated. For example, a library of molecules generated by a first round may itself be isolated and the isolated molecules used in a subsequent round. In this fashion, the number and type of motifs in the molecule may gradually be increased. Such iterative steps are of particular value for the generation of a selection of polypeptide species, each comprised of a different combination of protein modules. After each round of combination, the DNA molecules generated may be analysed as described below and promising candidate DNA molecules may then be selected and used in the next round of the method.

In general, the method according to the invention allows the production of DNA libraries of any desired size and diversity. Particular libraries include those, for example, in which the DNA has been rationally designed. Thus, SBs of a pre-determined length may be incorporated into a cut vector in sequential steps. This will be particularly advantageous when the DNA is intended to encode a multifunctional polypeptide sequence. For instance, in the Examples described herein relating to the construction of chimeric receptor sequences, the individual modules or domains that constitute the protein must be ordered appropriately, for example: binding components, extracellular spacer, transmembrane component, signalling component. Therefore, the individual SBs should be inserted in this order. Insertion in the correct orientation can be checked by restriction analysis or by nucleic acid sequencing.

In another example, the method allows the production of libraries generated from the random combination of SBs. In this method, a ligation reaction is carried out in which a large number of different SB components are employed, any of which may insert in either orientation. Depending upon the ligation reaction conditions, for example as

described above, a large number of different SB combinations is possible. Strings of multiple ligated SBs are then restricted with both the SB 5' restriction enzyme and the SB 3' restriction enzyme, to destroy combinations that contain one or more SBs in the incorrect orientation, prior to insertion of the assembled blocks into a cloning vector.

In the above examples and in general selective order of ligation of SBs, of either random or pre-determined length may be controlled by the selective use of kinases and phosphatases to add or remove phosphate groups from some or all of the SB components. As will be clear to the skilled artisan, a 5' terminal phosphate group must be donated by the ligating species in order that ligase-directed ligation is possible. In most instances, the vector should be phosphatased so as to prevent its self-ligation.

Thus according to a further aspect of the invention there is provided a DNA library and each nucleic acid component thereof generated by the method according to any one of the above-described aspects of the invention. The invention also provides a host cell transformed or transfected by such nucleic acid molecules and protein molecules expressed from said nucleic acid. Thus, the method of the invention also provides for the generation of a library of protein or polypeptide molecules.

The method of the invention is ideally suited to the generation of novel peptide or polypeptide compounds that are either wholly or partly derived from a combination of motifs, modules or domains. Such novel peptides, polypeptides, and indeed any protein library, may be expressed in a host cell(s), following transformation of the host with a nucleic acid (or library of nucleic acids) generated by the method of the invention. This may be achieved by culturing host cells under conditions that are well known in the art to be suitable for expression of a polypeptide from a nucleic acid (see for example: *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press; *DNA cloning: a practical approach, Volume II: Expression systems*, edited by D.M. Glover IRL Press, 1995; *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover IRL Press, 1995). Desirable peptide/polypeptide products may then be identified through any suitable screening process, and isolated, if required, by standard protein purification techniques that are well known in the art.

In this embodiment of the invention, the SBs are designed so as to encode an in-frame string of amino acids when the SBs are linked together in the correct orientation. In a preferred embodiment of the invention, the SBs are designed so that a stop codon is formed by the linkage of any two SBs in an incorrect orientation.

In the case of insertion of one or more SBs into a vector to express a polypeptide, the vector can be designed so that a stop codon is present after insertion of the last SB in the correct orientation. Preferably, the vector will have stop codons in all reading frames.

In the case of peptides, the SBs will encode short lengths of amino acid sequence, such as antigenic epitopes or signal sequences. However, the SBs may be significantly longer. Entire proteins may be assembled from component domains by the combination of SBs in either a random or a rational order. For example, many proteins are composed of domains that are identifiable by the presence of known consensus sequences or a prevalence of certain amino acid residues.

Binding proteins such as antibodies are examples of such proteins, being composed of constant and variable domains. Receptors are also usually composed of known domain structures, such as an extracellular binding component for recognition of ligand, a transmembrane region, a dimerisation or oligomerisation domain, linker domains, signalling domains and other intracellular domains. Combinations of synthetic and natural sequences can also be constructed. In this respect, spacer and linker domains may be used to maintain the steric configuration of the molecule, as and when appropriate for optimisation of function.

Other domains suitable for use in the invention may be derived from sources that will be clear to those of skill in the art and will include enzymatic effector domains such as protease, kinase or phosphatase domains.

Suitable applications for peptides formed by the combinatorial linkage of encoding SBs include many immunological applications. Most linear epitopes are fairly short in length, often comprising no more than 6-10 amino acid residues. It is envisaged that these epitopes may be combined to generate long stretches of sequence containing a number of different epitopes. Such a molecule might find application as a component of a vaccine, for example, if each of the epitopes is capable of eliciting a separate

immune response. For example, such a polyepitope peptide might contain known epitopes from bacterial pathogens that cause disease such as tetanus, cholera and diphtheria. Other epitopes that selectively stimulate populations of the immune system might be used, such as carrier peptides comprising epitopes recognised by T helper cells.

Short stretches of polypeptide comprising a number of peptides encoded by SBs may also be inserted into whole proteins or protein domains. For example, it may be desirable to include a plurality of signalling sequences into an existing protein. In this event, the assembled SB component would be inserted in-frame into the appropriate site of an existing nucleic acid sequence or gene. As exemplified below, one example of such an application is the creation of a synthetic signalling component from the assembly of multiple individual signalling motifs that have been derived from different naturally-occurring signalling regions. This ready-assembled component can then be inserted into a specific site in the sequence of a protein so as to confer on that protein the functional properties of each signalling motif.

Many similar applications may be envisaged, as will be clear to those of skill in the art.

As has been mentioned above, in one embodiment, the method of the present invention allows the design of nucleic acid molecules derived from a plurality of SBs that do not necessarily encode proteins, but which constitute regulatory elements that affect the rate of transcriptional or translational events. Each individual SB may encode an element such as a promoter element, a binding motif for a regulator or inhibitor protein, a response element, an enhancer element, a nuclease site, a hairpin motif, or any other element of a nucleic acid which may be transferred from its natural position in a gene and used in a different context without losing its function. For example, it may be desirable to create an improved transcriptional element to increase the transcription of a gene or to make such transcription dependent upon the presence of a particular activator or inhibitor compound.

Analysis of the DNA molecules that have been generated by the assembled SBs which have been produced in accordance with the present invention, may be by any suitable transcription or translation system. For example, when the method of the invention is being used to design an improved or modulated promoter or other regulatory element

affecting the levels of transcription, the analysis system may be a reporter plasmid, in which the assembled SBs are inserted upstream of an unrelated reporter gene so as to regulate their expression of the encoded reporter protein. Suitable reporter genes may be luciferase, β-galactosidase, alkaline phosphatase or green fluorescent protein (GFP).

An efficient promoter will give a high level of transcription that will be reflected in the levels of translated protein. Furthermore, the effect of an inhibitor or of an activator compound may be assessed by allowing transcription to take place in the presence of a suitable amount of that compound. Similarly, when the method of the invention is being used to generate DNA encoding a functional protein, the contents of any library may be assessed using an appropriate expression system with a suitable assay to screen for the desired, expressed, functional protein, for example, as described in the Examples herein.

The invention will now be described in further detail with specific reference to chimeric receptor molecules generated by the combination of SBs in a pBLUESCRIPT-based system. It will be appreciated that variation may be made from these specific Examples without departing from the scope of the invention.

All documents cited herein are incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic diagram showing system for assembly of SBs.

Figure 2: Cloning cassette for construction of chimeric receptors with synthetic signalling components.

Figure 3: Sequence of cloning cassette.

Figure 4: Oligonucleotide sequences for chimeric receptor construction.

Figure 5: Predicted amino acid sequence of SBs.

Figure 6: Level of expression and degree of activation of chimeric receptors containing synthetic signalling components by surface-bound antigen.

Figure 7: Activation of chimeric receptors containing synthetic signalling components by surface-bound antigen.

Figures 8-10: Activation of chimeric receptors containing synthetic signalling components by surface-bound antigen.

EXAMPLES

In these examples, we aim to construct a synthetic signalling component, from individual blocks of sequence, that is more potent than a naturally occurring signalling region for use in chimeric receptors. The individual sequence blocks are designed to include predicted signalling motifs from different naturally occurring signalling regions.

These sequence blocks (SBs) are constructed by annealing two synthetic strands of DNA (oligos) so that the 5' end forms a Bcl I overhang and the 3' end forms a BamH I overhang. Both these restriction site overhangs are compatible with a BamH I site in the cloning vector. Insertion of the SB in the correct orientation destroys the 5' BamH I site and retains a 3' BamH I site, allowing subsequent 3' insertion of SB(s). Insertion of the SB in the wrong orientation retains a 5 ' BamHI site and generates a 3' stop codon (Figure 1). In this example the SBs have 5' and 3' phosphate groups and the BamH I site in the construction vector (Figure 2) has its phosphate groups removed to prevent vector re-ligation in the absence of insert ligation.

EXAMPLE 1: CONSTRUCTION OF THE CLONING VECTOR, pHMF393

To facilitate construction of chimeric receptors with different binding, extracellular spacer, transmembrane and signalling components, a cloning cassette system was devised in pBluescript SK+ (Stratagene). This is a modification of our cassette system described in International Patent Specification No. WO 97/23613.

This new cassette system is shown in Figure 2. The binding component has 5' Not I and Hind III restriction sites and a 3' Spe I restriction site. The extracellular spacer has a 5' Spe I site (Thr, Ser) and a 3' Nar I site (Gly, Ala). The transmembrane component has a 5' Nar I site (Gly, Ala) and 3' Mlu I (Thr, Arg) and BamHI sites (Gly, Ser). The signalling component may be cloned into the BamHI site. Following this BamH I site there is a stop codon for transcription termination and there is an EcoRI site situated 3' of this for subsequent rescue of whole constructs.

To generate this cassette, a 200bp fragment was PCR assembled using oligos:- S0146, A6081, A6082 and A6083 (Figure 4). This fragment starts with a SpeI site and consists of the extracellular spacer h.CD28, the human CD28 transmembrane region, a stop codon and finishes with an EcoRI site (see Figure 3). This PCR fragment was then restricted with SpeI and EcoRI and substituted for the same fragment in our previously described cloning cassette system to join the binding component (International Patent No. WO 97/23613 Figure 2).

This cloning vector termed pHMF393 contains P67scFv/h.CD28/CD28TM and forms the base vector molecule into which synthetic signalling regions were built.

EXAMPLE 2 :CONSTRUCTION OF SEQUENCE BLOCKS (SBs)

Each sequence block was generated by annealing two oligos such that they have single-stranded overhangs forming half a Bcl I site at the 5' end and half a BamH I site at the 3' end. Oligos were annealed at a concentration of 1pmole/µl in a buffer consisting of :- 25mM NaCl, 12.5 mM Tris-HCl, 2.5mM $MgCl_2$, 0.25mM DTE, pH7.5 by heating in a boiling water bath for 5 minutes and then allowing the bath to cool slowly to room temperature.

The predicted amino acid sequences of these examples of SBs are shown in Figure 5

1) SB1

This sequence is based on the first ITAM of human TCR ζ and was constructed by annealing oligos A8816 and A8817 (Figure 4). Both these oligos have a 5' phosphate group.

2) SB2

This sequence is based on the second ITAM of human TCR ζ and was constructed by annealing oligos A8814 and A8815 (Figure 4). Both these oligos have a 5' phosphate group.

3) SB3

This sequence is based on the third ITAM of human TCR ζ and was constructed by annealing oligos A8812 and A8813 (Figure 4). Both these oligos have a 5' phosphate group.

4) SB4

This sequence is based on the ITAM of the γ chain of human FcεR1 and was constructed by annealing oligos A8810 and A8811 (Figure 4). Both these oligos have a 5' phosphate group.

5) SB4*

This sequence was originally generated in error by mis-annealment of the above oligos but was subsequently made by annealing oligos A8810B and A8811B (Figure 4). Both these oligos have a 5' phosphate group.

6) SB5

This sequence is based on the ITAM of the β chain of human FcεR1 and was constructed by annealing oligos A9000 and A9001 (Figure 4). Both these oligos have a 5' phosphate group.

7) SB6

This sequence is based on the ITAM of the γ chain of human CD3 and was constructed by annealing oligos A9002 and A9003 (Figure 4). Both these oligos have a 5' phosphate group.

8) SB7

This sequence is based on the ITAM of the δ chain of human CD3 and was constructed by annealing oligos A9004 and A9005 (Figure 4). Both these oligos have a 5' phosphate group.

9) SB8

This sequence is based on the ITAM of the ε chain of human CD3 and was constructed by annealing oligos A9006 and A9007 (Figure 4). Both these oligos have a 5' phosphate group.

10) SB9

This sequence is based on the ITAM of human CD5 and was constructed by annealing oligos A9008 and A9009 (Figure 4). Both these oligos have a 5' phosphate group.

11) SB10

This sequence is based on the ITAM of human CD22 and was constructed by annealing oligos A9010 and A9011 (Figure 4). Both these oligos have a 5' phosphate group.

12) SB11

This sequence is based on the ITAM of human CD79a and was constructed by annealing oligos A9012 and A9013 (Figure 4). Both these oligos have a 5' phosphate group.

13) SB12

This sequence is based on the ITAM of human CD79b and was constructed by annealing oligos A9014 and A9015 (Figure 4). Both these oligos have a 5' phosphate group.

14) SB13

This sequence is based on the ITAM of human CD66d and was constructed by annealing oligos A9016 and A9017 (Figure 4). Both these oligos have a 5' phosphate group.

15) SB28

This sequence is based on the co-stimulation motif of human CD28 and was constructed by annealing oligos A9018 and A9019 (Figure 4). Both these oligos have a 5' phosphate group.

16) SB29

This sequence is based on the co-stimulation motif of human CD154 and was constructed by annealing oligos A9020 and A9021 (Figure 4). Both these oligos have a 5' phosphate group.

EXAMPLE 3 CONSTRUCTION OF MUTATED SEQUENCE BLOCKS

Exemplification of mutation of SB1.

SB1, based on the first ITAM of TCR ζ, has the naturally occurring amino acid sequence of: QNQLY**NEL**NLGRREEY**DVL**DKRRGRDPEM. Degenerate oligonucleotides (D7001 and D7002) were designed to alter the three amino acid residues following each of the ITAM-defining tyrosine residues (i.e. the six residues highlighted in bold, above). The oligonucleotides were designed such that any residue could be introduced at the first two positions following the tyrosine, but only leucine, or isoleucine, or valine would be introduced at the third position. Thus, mutated SBs with the following sequence would be generated (where X is any amino acid):

```
          I          I
GSQNQLYXXLNLGRREEYXXLDKRRGRDPEMGS
          V          V
```

Degenerate oligonucleotides D7001 and D7001 were annealed at a concentration of 1 pmole/μl in a buffer consisting of 25mM NaCl, 12.5mM Tris.HCl, 2.5mM $MgCl_2$, 0.25mM DTE, pH7.5, by heating in a boiling water bath for 5 minutes and then allowing them to cool slowly to room temperature. Approximately 1 pmole of annealed oligonucleotides were mixed with approximately 1 ng of BamHI-digested pBluescript, T4 DNA ligase and ATP in a 10μl reaction volume and ligation performed under the manufacturer's recommended conditions. Competent *Escherichia coli*, strain XL1-Blue, were transformed with 2μl of ligation reaction, and plated onto LB agar containing ampicillin. Ampicillin resistant colonies were picked and grown up in L-broth containing ampicillin, and plasmid DNA prepared. The presence and sequence of any insert in the pBluescript vector was confirmed by sequencing with oligonucleotides corresponding to regions 5' and 3' to the BamHI site in the vector. Representative

examples of mutant SBs generated using this method with degenerate oligonucleotides D7001 and D7002 are given as SBW1A, SBW1B, SBW1C and SBW1D in Table 1.

Table 1. Examples of mutant sequence blocks generated using degenerate oligonucleotides D7001 and D7002.

Sequence Block	Amino Acid Sequence
SBW1A	GSQNQL**YPPL**NLGRREE**YRPL**DKRRGRDPEMGS
SBW1B	GSQNQL**YGGL**NLGRREE**YGKI**DKRRGRDPEMGS
SBW1C	GSQNQL**YGAV**NLGRREE**YTGV**DKRRGRDPEMGS
SBW1D	GSQNQL**YTGI**NLGRREE**YGTV**DKRRGRDPEMGS

5

EXAMPLE 4: CONSTRUCTION OF CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING REGIONS BY SEQUENTIAL ADDITION

The vector, pHMF393 was digested with the restriction enzyme BamH I under the manufacturer's recommended conditions and then treated with alkaline phosphatase for 10 minutes at 37°C in the same buffer. The linearised vector fragment was eluted from an agarose gel and purified. Approximately 1 ng of vector fragment was ligated to approximately 1 pmole of SB or mixture of SBs using T4 ligase and ATP under the manufacturer's recommended conditions in a 10 µl reaction. 2 µl of the ligation reaction was transformed into XL-1 blue competent *E. coli* and plated onto L-broth/Ampicillin plates. Ampicillin resistant colonies were picked and grown up in L-broth containing Ampicillin, and plasmid DNA prepared. Correct orientation of the inserted SB was established by digesting the DNA with BamH I and an enzyme within the Vector (Nar I). Optionally before picking, colonies were screened for the presence of inserted SBs by PCR using oligos corresponding to regions 5' and 3' to the BamH I site in the vector. Positive colonies were then grown up in L-broth containing Ampicillin and plasmid DNA prepared. Correct orientation of the inserted SB was established by digesting the DNA with BamH I and an enzyme within the Vector (Nar I).

Correct plasmids were then digested with BamH I, treated with alkaline phosphatase, gel purified and ligated to an SB, or mixture of SBs, as described above to insert a second SB. Colonies were screened again in the same way to find vectors with a second SB in the correct orientation. These vectors were then put through further rounds of digestion, purification, ligation and screening, as desired, to generate the required number of SBs in the correct orientation in a vector.

Specific example : (see Table 2)

P67/h.CD28/CD28TM/SB2.SB1.SB3.SB1 (pHMF369) was constructed by the following steps of sequential addition:-

ligation of vector pHMF393 to SB2 to generate pHMF403

ligation of vector pHMF403 to SB1 to generate pHMF410

ligation of vector pHMF410 to SB3 to generate pHMF432

ligation of vector pHMF432 to SB1 to generate pHMF469.

EXAMPLE 5: CONSTRUCTION OF CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING REGIONS BY MULTIPLE ADDITION

Construction of synthetic signalling regions with random combinations of SBs by adding mixtures of more than one SB at a time was done by the following methods:-

a) SBs were ligated to each other in the absence of vector and then digested with both Bcl I and BamH I to cut SBs ligated to each other in incorrect orientations. If a specific number of SBs were required, then the desired size fragments were gel eluted and purified; if not, the ligated SB fragments were purified from the restriction enzymes and then ligated to the digested, phosphatased and purified vector as in Example 3. Ampicillin-resistant colonies were then generated and screened as in Example 3 to select vectors with more than one SB in the correct orientation in a vector.

b) SBs were ligated to the digested, phosphatased and purified vector and then digested with both Bcl I and BamH I to cut SBs ligated to each other and to the vector in incorrect orientations. Fragments larger than the unligated vector were gel purified and then ligated to recircularise. Ampicillin-resistant colonies were then generated and screened as in Example 3, to select vectors with more than one SB in the correct orientation in a vector.

c) SBs were ligated to the digested, phosphatased and purified vector at a ratio of SB to vector that favoured the insertion of multiple SBs; Ampicillin resistant colonies were then generated and screened as in Example 3 to select vectors with more than one SB in the correct orientation in a vector.

It was found that for maximum efficiency it was desirable to use method c and establish a titration of SB for each vector. A de-stimulation of colony number from vector only controls was a good indication of insertion of multiple SBs. It was also found to be more efficient to perform two rounds of ligation and screening for correct insertion of a low number of SBs rather than one round of ligation and screening for many SBs in the correct orientation. Statistically; adding 1 SB, 50% should be in the correct orientation; adding 2 SBs, 25% should be in the correct orientation but when 4SBs are added at one time only 6% of vectors would have all 4 SBs in the desired orientation.

In the case of multiple rounds of multiple insertion, mixtures of both vector and SBs were ligated to each other to increase diversity of the library produced.

Specific examples: (see Table 2)
P67/h.CD28/CD28TM/SB11.SB5.SB10.SB9 (pHMF537);
P67/h.CD28/CD28TM/SB4.SB7.SB10 (pHMF538);
P67/h.CD28/CD28TM/SB4.SB3 (pHMF539); and
P67/h.CD28/CD28TM/SB4.SB1 (pHMF540) were constructed by ligating a mixture of linearised and phophatased vectors already containing one SB :-
pHMF 403,404,405, 406, 515 and 516 to a mixture of SBs :-
SB1,SB2,SB3,SB4,SB4*,SB5,SB6,SB7,SB8,SB9,SB10,SB11,SB12,SB13 and SB28.
P67/h.CD28/CD28TM/SB2.SB1.SB1 (pHMF529);
P67/h.CD28/CD28TM/SB2.SB4.SB5 (pHMF530);
P67/h.CD28/CD28TM/SB11.SB4*.SB3 (pHMF531); and
P67/h.CD28/CD28TM/SB11.SB4*.SB10* (pHMF532) were constructed by ligating a mixture of linearised and phophatased vectors already containing two SBs :-pHMF 408,410,412, 518, 519, 520, 521 and 522 to a mixture of SBs :-
SB1,SB2,SB3,SB4,SB4*,SB5,SB6,SB7,SB8,SB9,SB10,SB11,SB12,SB13 and SB28.

EXAMPLE 6: ANALYSIS OF RECEPTORS

a) Construction of expression plasmids

The chimeric receptor constructs were subcloned from pBluescript KS+ into the expression vector pEE6hCMV.ne (C.R. Bebbington (1991), Methods 2, 136-145) on a HindIII to EcoRI restriction fragment. The expression vector with no chimeric receptor genes is used as a negative control in subsequent experiments.

b) Stable transfection into Jurkat E6.1 cells

The expression plasmids were linearised and transfected into Jurkat E6.1 cells (ECACC) by electroporation using a Bio-rad Gene Pulser. 10µg of DNA per 2.5 $X10^6$ cells was given two pulses of 1000V, 3µF in 1ml PBS. Cells were left to recover overnight in non-selective medium before being selected and cultured in medium

supplemented with the antibiotic G418 (Sigma) at 1.5mg/ml. After approximately four weeks the cells were ready for analysis.

c) FACS analysis of surface expression

Approximately $5X10^5$ Jurkat cells were stained with 1μg/ml FITC labelled antigen, CD33 extracellular region. Fluorescence was analysed by a FACScan cytometer (Becton Dickinson).

d) IL-2 production analysis of function

$2X10^5$ cells were incubated at 37°C/8% CO_2 for 20 hours in 96 well plates with CD33 expressing HL60 target cells at an effector : target ratio of 1:1. Cell supernatants were then harvested and assayed for human IL-2 (R & D Systems Quantikine kit).

EXAMPLE 7 : RESULTS

The library of synthetic signalling regions produced is listed in Table 2. These were constructed by the methods described in example 3 and example 4. All signalling regions were sequenced prior to analysis and some additional diversity of the library was found to arise from mis-annealment and recombination events and these are listed in the notes to Table 2.

This diverse library of synthetic signalling regions were demonstrated to function specifically in response to antigen (see Figures 6 to 10).

Table 2

pBluescript					EE6hC MVNE / Jurkat line	NOTES
	Binding	Spacer	Trans-membrane	Signalling Region		
pHMF403	hP67scFv	h.CD28	CD28	SB1	pHMF/ J.434	
404	hP67scFv	h.CD28	CD28	SB2	435	
405	hP67scFv	h.CD28	CD28	SB4	436	
406	hP67scFv	h.CD28	CD28	SB3	437	

407	hP67scFv	h.CD28	CD28	SB1.SB1	438	
408	hP67scFv	h.CD28	CD28	SB1.SB2	439	
409	hP67scFv	h.CD28	CD28	SB1.SB3	440	
410	hP67scFv	h.CD28	CD28	SB2.SB1	441	
411	hP67scFv	h.CD28	CD28	SB2.SB3	442	
412	hP67scFv	h.CD28	CD28	SB2.SB4	443	
427	hP67scFv	h.CD28	CD28	SB1.SB4	444	
428	hP67scFv	h.CD28	CD28	SB2.SB4*	445	1
429	hP67scFv	h.CD28	CD28	SB4.SB4*	446	1
430	hP67scFv	h.CD28	CD28	SB3.SB4*	447	1
431	hP67scFv	h.CD28	CD28	SB1.SB2.SB3	448	
432	hP67scFv	h.CD28	CD28	SB2.SB1.SB3	449	
433	hP67scFv	h.CD28	CD28	SB2.SB4.SB3	500	
469	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB1*	475	2
470	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB2	476	
471	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB3	477	
472	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB4	478	
473	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB1	479	3
474	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB2	480	3
501	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB28	507	3
502	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB1*.SB28		
503	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB2.SB28	508	
504	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB29	509	
505	hP67scFv	h.CD28	CD28	SB2.SB1.SB3.SB1*.SB29		
506	hP67scFv	h.CD28	CD28	SB2.SB4.SB3.SB2.SB29	510	3
511	hP67scFv	h.CD28	TM24	SB2.SB1.SB3.SB1*	512	2
513	hP67scFv	h.CD28	CD28	SB2.SB1.SB28	514	
515	hP67scFv	h.CD28	CD28	SB11	523	
516	hP67scFv	h.CD28	CD28	SB28		
517	hP67scFv	h.CD28	CD28	SB3'.SB4*		1,4
518	hP67scFv	h.CD28	CD28	SB11.SB4*	524	1
519	hP67scFv	h.CD28	CD28	SB3.SB3	525	
521	hP67scFv	h.CD28	CD28	SB4*.SB1	527	1,5
522	hP67scFv	h.CD28	CD28	SB4*.SB3	528	1
529	hP67scFv	h.CD28	CD28	SB2.SB1.SB1	533	
530	hP67scFv	h.CD28	CD28	SB2.SB4.SB5	534	
531	hP67scFv	h.CD28	CD28	SB11.SB4*.SB3	535	1
532	hP67scFv	h.CD28	CD28	SB11.SB4*.SB10*	536	1,6
537	hP67scFv	h.CD28	CD28	SB11.SB5.SB10.SB9	559	
538	hP67scFv	h.CD28	CD28	SB4.SB7.SB10	560	
539	hP67scFv	h.CD28	CD28	SB4.SB3		
540	hP67scFv	h.CD28	CD28	SB4.SB1		
541	hP67scFv	h.CD28	CD28	SB1.SB3		
542	hP67scFv	h.CD28	CD28	SB28.SB1	561	
543	hP67scFv	h.CD28	CD28	SB11.SB7	562	
544	hP67scFv	h.CD28	CD28	SB3.SB13	563	
545	hP67scFv	h.CD28	CD28	SB28.SB4*	564	1

547	hP67scFv	h.CD28	CD28	SB2.SB1.SB2	565	
549	hP67scFv	h.CD28	CD28	SB4*.SB3.SB3	567	1
550	hP67scFv	h.CD28	CD28	SB1.SB2.SB9	568	
551	hP67scFv	h.CD28	CD28	SB1.SB2.SB12	569	
552	hP67scFv	h.CD28	CD28	SB4*'.SB1.SB1	570	1,5
553	hP67scFv	h.CD28	CD28	SB3.SB3.SB4*	571	1
554	hP67scFv	h.CD28	CD28	SB4*.SB1.SB12	572	1
555	hP67scFv	h.CD28	CD28	SB2.SB4.SB28	573	
556	hP67scFv	h.CD28	CD28	SB3.SB3.SB7	574	
557	hP67scFv	h.CD28	CD28	SB11.SB4*.SB10	575	1
558	hP67scFv	h.CD28	CD28	SB1.SB4*.SB3	576	1
577	hP67scFv	h.CD28	CD28	SB28.SB4*.SB2	589	1
578	hP67scFv	h.CD28	CD28	SB28.SB1.SB3	590	
579	hP67scFv	h.CD28	CD28	SB28.SB4*.SB4*	591	1
580	hP67scFv	h.CD28	CD28	SB28.SB1.SB28	592	
581	hP67scFv	h.CD28	CD28	SB28.SB1.SB2	593	
582	hP67scFv	h.CD28	CD28	SB28.SB1.SB13	594	
583	hP67scFv	h.CD28	CD28	SB28.SB3*	595	
584	hP67scFv	h.CD28	CD28	SB28.SB13	596	
585	hP67scFv	h.CD28	CD28	SB28.SB10	597	
586	hP67scFv	h.CD28	CD28	SB28.SB4*.SB2.SB4	598	1
587	hP67scFv	h.CD28	CD28	SB28.SB1.SB4"	599	7
608	hP67scFv	h.CD28	CD28	SB28.SB2	612	
609	hP67scFv	h.CD28	CD28	SB11.SB2	613	
610	hP67scFv	h.CD28	CD28	SB28.SB2.SB1	614	
611	hP67scFv	h.CD28	CD28	SB28.SB2.SB4	615	

NOTES TO TABLE 2

1) SB4* is a single amino acid different from SB4, initially generated by mis-annealment of oligos but subsequently deliberately generated by annealing oligos A8810B and A8811B (see Figure 4) due to enhanced activity.

SB4* :- GSYEKSDGVYTGLSTRNQETYDTLKHEKPGS

2) SB1* is a truncated version of SB1 generated by a recombination event during cloning.

SB1* :- GSGQNQLYNELNLGRREEYDVLAK

3) R to G change at the 5' end of SB3

4) A to T change at the 3' end of SB3

5) K to R change at the 5' end of SB4*

6) SB10* is a truncated ,version of SB10 with an altered 3' end.

SB10* :- GSPPRTCDDTVTYSALHKRQVGDYENVIPER

7) S to L change at the 5' end of SB4 and a S to G change in the middle of SB4.

SB4" :- GLYEKSDGVYTGLGTRNQETYETLKHEKPGS

CLAIMS

1. A method of generating a library of DNA molecules of varying length and sequence in a desired orientation comprising the steps of:

a) providing a mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which are compatible to each other and correspond to the cleavage products of different but compatible restriction enzymes; and

b) allowing ligation to take place, wherein ligation of said double-stranded DNA molecules in desired orientations generates molecules that are not cut by either of said restriction enzymes whereas ligation in undesired orientations generates molecules that retain at one or more ligation points a restriction site that is recognised by one of said restriction enzymes; and

c) cutting the ligated DNA molecules with one or both of said restriction enzymes such that only molecules that are ligated in undesired orientations are cut, leaving a library of DNA molecules of varying length and sequence in a desired orientation.

2. A method of generating a library of DNA molecules of varying length and sequence in a desired orientation in a vector comprising the steps of:

a1) cutting a double-stranded DNA vector molecule with a first restriction enzyme;

a2) adding to the cut vector molecule a mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which correspond to the cleavage products of different but compatible restriction enzymes, one of said restriction enzymes being said first restriction enzyme;

b) allowing ligation to take place;

c) cutting the ligated DNA molecules with at least said first restriction enzyme such that molecules that are ligated in an incorrect orientation in the vector are cut out of the vector; and optionally

d) repeating steps (a2) to (c); to leave a library of DNA molecules of varying length and sequence in a desired orientation in a vector.

3. A method of generating a library of DNA molecules of varying length and sequence in a desired orientation on a solid phase comprising the steps of:

a1) providing a solid phase to which is attached a first double-stranded DNA molecule which has an end corresponding to the cleavage product of a first restriction enzyme;

a2) adding to said solid phase a mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which correspond to the cleavage product of a different but compatible restriction enzyme, one of said restriction enzymes being said first restriction enzyme;

b) allowing ligation to take place, wherein ligation of said double-stranded DNA molecules in a correct orientation generates a molecule that is not cut by either of said restriction enzymes, whereas ligation in an incorrect orientation retains at one or more ligation points a restriction site that is recognised by one of said restriction enzymes; and

c) cutting the ligated DNA molecules with one or both of said restriction enzymes such that only molecules that are ligated in an incorrect orientation are cut, thus leaving a library of DNA molecules of varying length and sequence in a desired orientation.

4. A method according to claim 3 wherein said solid phase is a bead.

5. A method according to claims 1 to 4 wherein each double-stranded DNA molecule has single-stranded cohesive ends.

6. A method according to claim 5, wherein said first double-stranded DNA molecule contains a unique recognition site for a restriction enzyme that does not cut said second double-stranded DNA molecules to allow the subsequent insertion of the ligated DNA molecules into a vector.

7. A method according to claim 6 additionally comprising the step of cutting at said unique restriction site and inserting said ligated DNA molecule into a vector.

8. A method according to any one of claims 3-7 wherein in step a), said mixture comprises a library produced according to claim 1.

9. A method according to any of the preceding claims further comprising the steps of:

e) isolating correctly ligated DNA molecules;

f) adding said isolated DNA molecules to a further mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which correspond to the cleavage product of different but compatible restriction enzymes and repeating steps b) and c);

and optionally,

g) repeating steps e) and f); to leave a library of DNA molecules of varying length and sequence in a desired orientation.

10. A method according to any one of the preceding claims further comprising the step of selecting the ligated DNA molecules for a desired length after step b) and/or after step c).

11. A method according to any one of the preceding claims wherein the library of DNA molecules produced is subsequently cut with a third restriction enzyme and added to a further mixture of double-stranded DNA molecules, each of said molecules having 5' and 3' ends which correspond to the cleavage product of said third enzyme and the cleavage product of a restriction enzyme that is different but compatible with said 3rd restriction enzyme, ligation is allowed to take place, and the ligated DNA molecules are then cut with at least said 3rd

restriction enzyme such that molecules that are ligated in an incorrect orientation are cut out.

12. A method according to any one of the preceding claims wherein said mixture of double-stranded DNA molecules is a mixtureof annealed single-stranded oligonucleotides.

13. A method according to any one of the preceding claims wherein said mixture of double-stranded DNA molecules is a mixture of annealed single-stranded oligonucleotides with 5' phosphate groups.

14. A method according to any one of the preceding claims wherein said mixture of double-stranded DNA molecules is a mixture of annealed single-stranded oligonucleotides including mis-matched base pairs.

15. A method according to any one of the preceding claims wherein said mixture of double-stranded DNA molecules is generated by amplifying a DNA molecule using PCR and cutting with appropriate restriction enzymes.

16. A method according to any one of claims 1 to 11 wherein said mixture of double-stranded DNA molecules is generated by cutting genomic or cDNA with appropriate restriction enzyme.

17. A method according to any one of claims 1 to 13 wherein at least one of said double-stranded DNA molecules carries a mutation.

18. A method according to claim 17 wherein said double-stranded DNA molecule(s) carrying a mutation have been generated by:

combining degenerate oligonucleotides under conditions such that substantially complementary oligonucleotides anneal to form a plurality of double-stranded DNA molecules, ligating said plurality of double-stranded DNA molecules into a vector, transforming the modified vector molecules into a host cell, culturing said transformed cell under conditions suitable for growth and cell division and isolating said mutant double-stranded molecule(s) from said host cell.

19. A method according to claim 18, wherein the sequences of at least one of the degenerate oligonucleotides are based on a parent nucleic acid.

20. A method according to claim 19, wherein said parent nucleic acid encodes a polypeptide.

21. A method according to any one of claims 18-20, wherein at least one of said complementary oligonucleotides exhibits degeneracy at a ratio equal to or less than 1 in every 5 nucleotides.

22. A method according to any one of claims 18-21, wherein degeneracy is clustered in groups of 3 adjacent nucleotides, and/or any 2 out of 3 adjacent nucleotides, and/or any 1 out of 3 adjacent nucleotides.

23. A method according to claim 22, wherein said 3 adjacent nucleotides encode an amino acid of the polypeptide encoded by any one of the plurality of double-stranded DNA molecules, and/or an amino acid of the polypeptide encoded by the parent nucleic acid.

24. A method according to any one of claims 18 to 23 wherein the degeneracy at one or more nucleotide positions is generated through the inclusion of all of A, C, G, or T, or alternatively I, at the desired site of mutation.

25. A method according to any one of claims 18 to 23, wherein the degeneracy at one or more nucleotide positions is limited to a selection of bases.

26. A method according to any one of claims 18-25, wherein each of a pair of substantially complementary oligonucleotides exhibits degeneracy at a corresponding position when the two oligonucleotides are annealed.

27. A double-stranded vector molecule according to claim 2a1) wherein the restriction site of said first enzyme is adjacent to a stop codon in one or all these reading frames.

28. A library of DNA molecules generated by a method according to any one of claims 1-26.

29. A nucleic acid isolated from a library of DNA molecules according to claim 28.

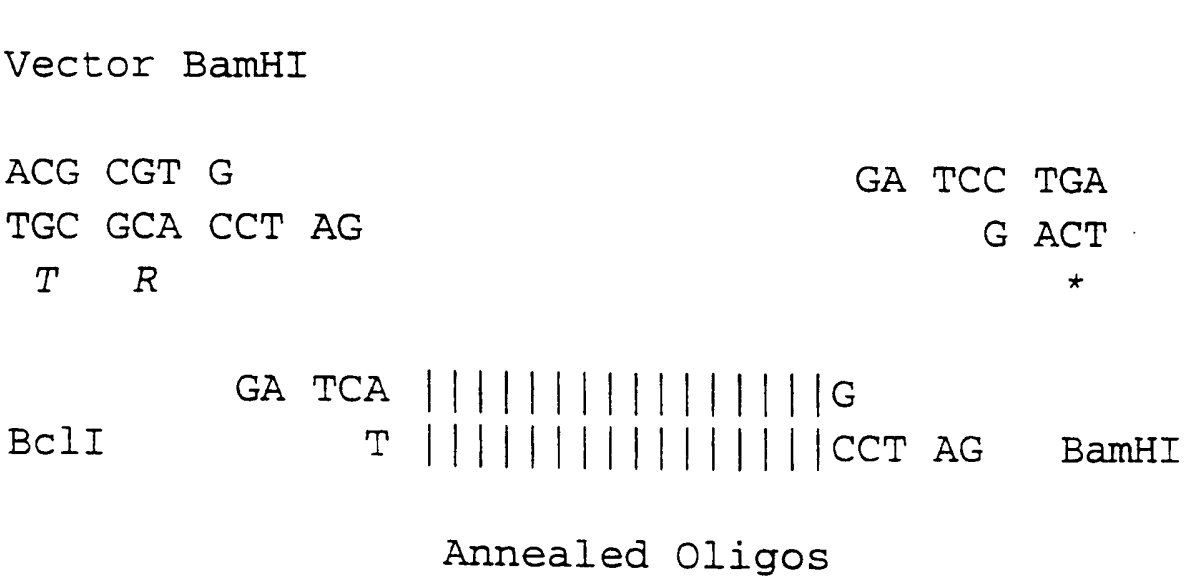
30. A polypeptide encoded by a nucleic acid of claim 29.

31. A host cell transformed with a nucleic acid according to claim 29.

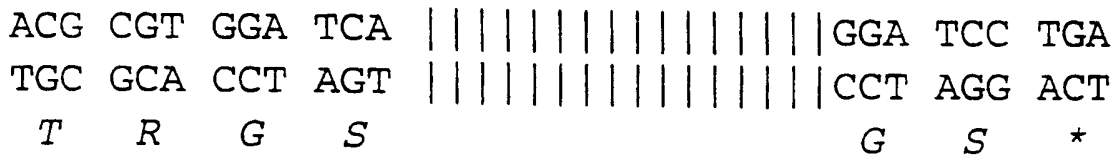
32. A library of protein or polypeptide molecules encoded by a library of DNA molecules according to claim 28.

FIG. 1

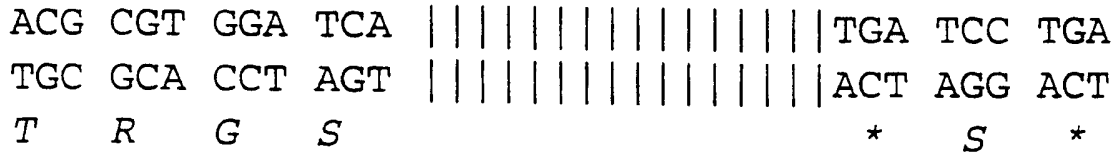
SEQUENTIAL SINGLE SEQUENCE ADDITION



Correct Orientation:



Wrong Orientation:



MULTIPLE ADDITION

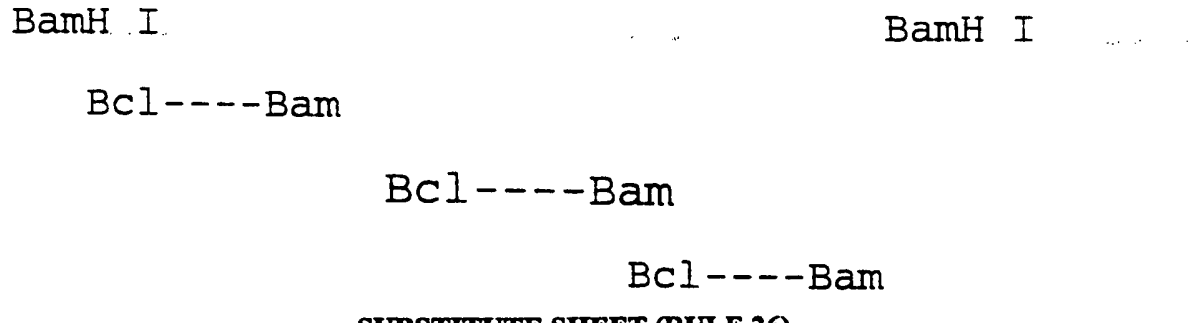


FIG. 2
CLONING CASSETTE FOR CONSTRUCTION OF CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

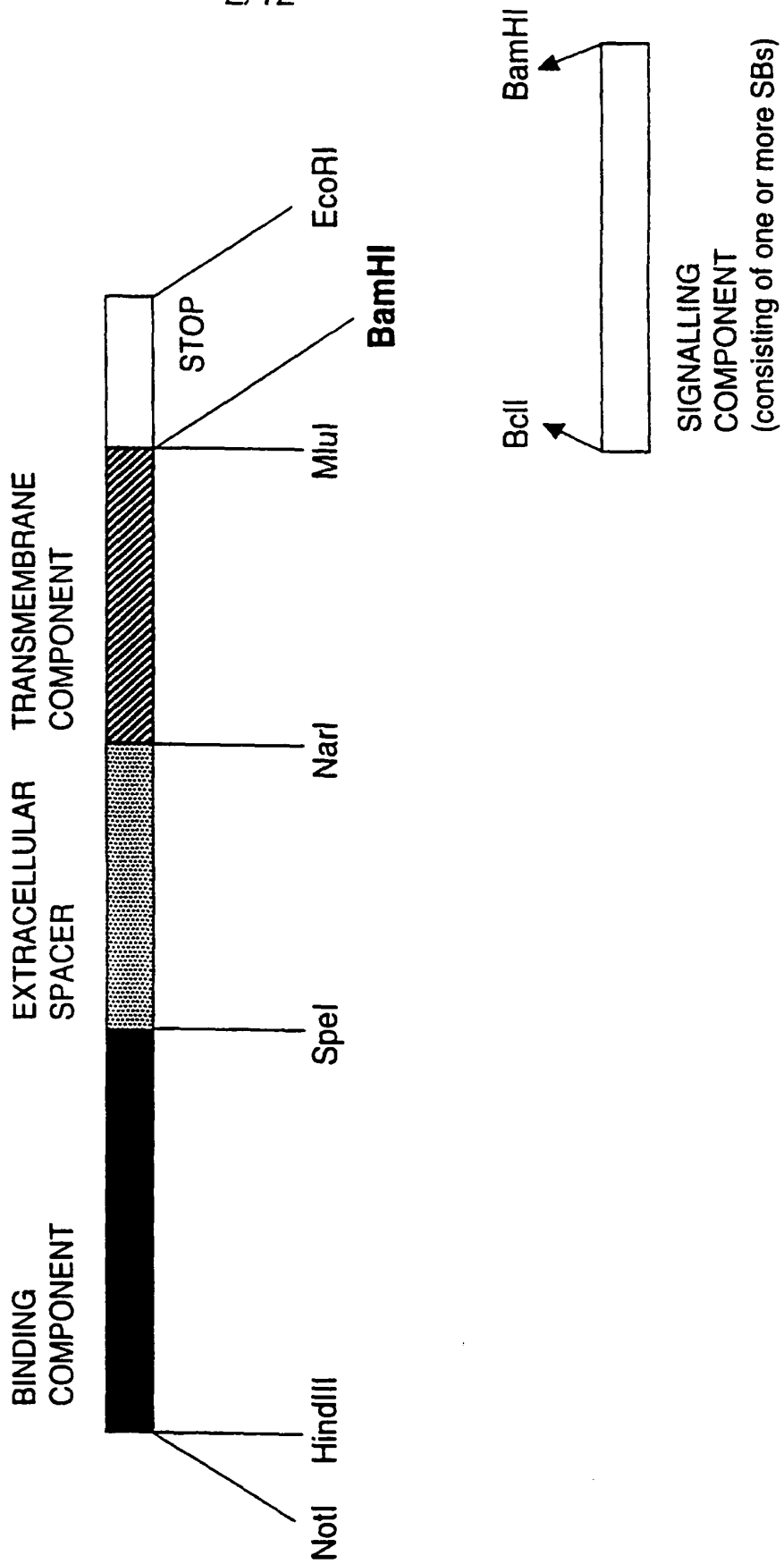


FIG. 3

Range: 1 to 200

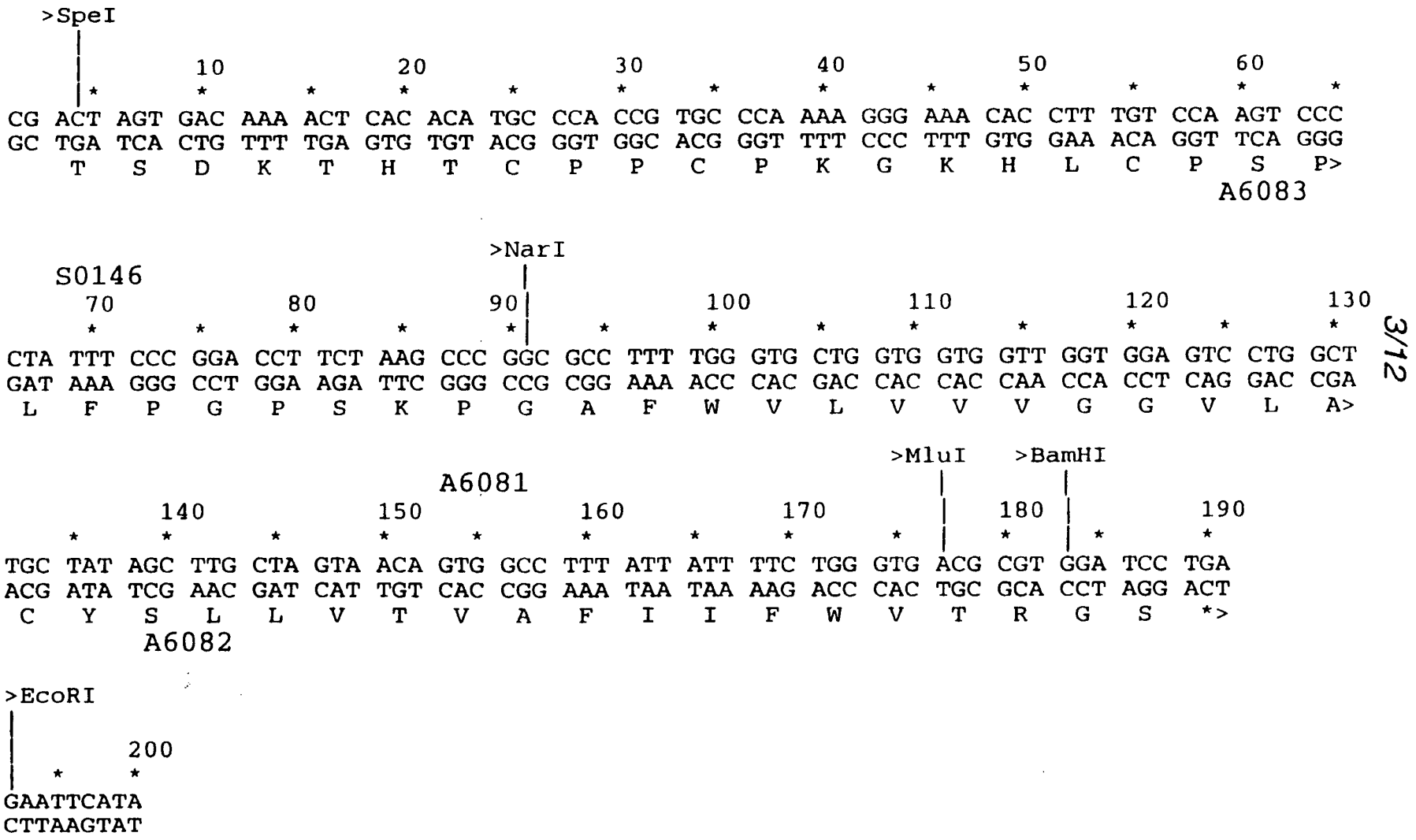


FIG. 4

OLIGONUCLEOTIDE SEQUENCES FOR CHIMERIC RECEPTOR CONSTRUCTION

All oligos are listed in the 5' to 3' orientation

S0146:CGACTAGTGACAAAACTCACACATGCCCACCGTGCCCAAAAGGGAAACACCT
TTGTCCAACTCCC

A6081:GCCTTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGC
TAGTAACAGTG

A6082:TATGAATTCTCAGGATCCACGCGTCACCCAGAAAATAATAAAGGCCACTGTTA
CTAGCAAGCTATAG

A6083:CACCACCAGCACCCAAAAGGCGCCGGGCTTAGAAGGTCCGGGAAATAGGGG
ACTTGGAC

A8810:GATCCTGGTTTCTCATGCTTCAGAGTCTCGTAAGTCTCCTGGTTCCTGGTGCT
CAGGCCCGTGTAACACCATCTGATTTCTCATAT

A8810B:GATCCTGGTTTCTCATGCTTCAGAGTATCGTAAGTCTCCTGGTTCCTGGTGC
TCAGGCCCGTGTAACACCATCTGATTTCTCATAT

A8811:GATCATATGAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAACCA
GGAGACTTACGAGACTCTGAAGCATGAGAAACCAG

A8811B:GATCATATGAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAACC
AGGAGACTTACGATACTCTGAAGCATGAGAAACCAG

A8812:GATCCGGCCTGCATGTGAAGGGCGTCGTAGGTGTCCTTGGTGGCTGTACTGA
GACCCTGGTAAAGGCCATCGTGCCCCTGTCCCCTT

A8813:GATCAAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCA
CCAAGGACACCTACGACGCCCTTCACATGCAGGCCG

A8814:GATCCGCGCTCGCCTTTCATCCCAATCTCACTGTAGGCCTCCGCCATCTTATC
TTTCTGCAGTTCATTGTACAGGCCTTCCTGAGGGTTCTTCCTT

A8815:GATCAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAA
GATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCG

A8816:GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCCAAAACATCGTACTCCTC
TCTTCGTCCTAGATTGAGCTCGTTATAGAGCTGGTTCTGGCCT

A8817:GATCAGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGA
GTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGG

A9000:GATCAGGAAACAAGGTTCCAGAGGATCGTGTTTATGAAGAATTAAACATATAT
TCAGCTACTTACAGTGAGTTGGAAGACCCAGGGGAAATGTCTCCTG

FIG. 4(contd.)

A9001:GATCCAGGAGACATTTCCCCTGGGTCTTCCAACTCACTGTAAGTAGCTGAATA
TATGTTTAATTCTTCATAAACACGATCCTCTGGAACCTTGTTTCCT

A9002:GATCAAAGCAGACTCTGTTGCCCAATGACCAGCTCTACCAGCCCCTCAAGGA
TCGAGAAGATGACCAGTACAGCCACCTTCAAGGAAACCAGTTGAGGG

A9003:GATCCCCTCAACTGGTTTCCTTGAAGGTGGCTGTACTGGTCATCTTCTCGATC
CTTGAGGGGCTGGTAGAGCTGGTCATTGGGCAACAGAGTCTGCTTT

A9004:GATCAGCTCTGTTGAGGAATGACCAGGTCTATCAGCCCCTCCGAGATCGAGA
TGATGCTCAGTACAGCCACCTTGGAGGAAACTGGGCTCGGAACAAGG

A9005:GATCCCTTGTTCCGAGCCCAGTTTCCTCCAAGGTGGCTGTACTGAGCATCATC
TCGATCTCGGAGGGGCTGATAGACCTGGTCATTCCTCAACAGAGCT

A9006:GATCACAAAACAAGGAGAGGCCACCACCTGTTCCCAACCCAGACTATGAGCC
CATCCGGAAAGGCCAGCGGGACCTGTATTCTGGCCTGAATCAGAGACGCATCG

A9007:GATCCGATGCGTCTCTGATTCAGGCCAGAATACAGGTCCCGCTGGCCTTTCC
GGATGGGCTCATAGTCTGGGTTGGGAACAGGTGGTGGCCTCTCCTTGTTTTGT

A9008:GATCACACGTGGATAACGAATACAGCCAACCTCCCAGGAACTCCCGCCTGTC
AGCTTATCCAGCTCTGGAAGGGGTTCTGCATCGCTCCG

A9009:GATCCGGAGCGATGCAGAACCCCTTCCAGAGCTGGATAAGCTGACAGGCGG
GAGTTCCTGGGAGGTTGGCTGTATTCGTTATCCACGTGT

A9010:GATCACCTCCCCGGACCTGCGATGACACGGTCACTTATTCAGCATTGCACAA
GCGCCAAGTGGGCGACTATGAGAACGTCATTCCAGATTTTCCAGAAGATGAGG

A9011:GATCCCTCATCTTCTGGAAAATCTGGAATGACGTTCTCATAGTCGCCCACTTG
GCGCTTGTGCAATGCTGAATAAGTGACCGTGTCATCGCAGGTCCGGGGAGGT

A9012:GATCAGAATATGAAGATGAAAACCTTTATGAAGGCCTGAACCTGGACGACTGC
TCCATGTATGAGGACATCTCCCGGGGCCTCCAGGGCACCTACCAGGATGTGG

A9013:GATCCCACATCCTGGTAGGTGCCCTGGAGGCCCCGGGAGATGTCCTCATACA
TGGAGCAGTCGTCCAGGTTCAGGCCTTCATAAAGGTTTTCATCTTCATATTCT

A9014:GATCAAAGGCTGGCATGGAGGAAGATCACACCTACGAGGGCCTGGACATTGA
CCAGACAGCCACCTATGAGGACATAGTGACGCTGCGGACAGGGGAAGTGG

A9015:GATCCCACTTCCCCTGTCCGCAGCGTCACTATGTCCTCATAGGTGGCTGTCT
GGTCAATGTCCAGGCCCTCGTAGGTGTGATCTTCCTCCATGCCAGCCTTT

A9016:GATCACCCCTACCCAACCCCAGGACAGCAGCTTCCATCTATGAGGAATTGCTA
AAACATGACACAAACATTTACTGCCGGATGGACCACAAAGCAGAAGTGGCTG

A9017:GATCCAGCCACTTCTGCTTTGTGGTCCATCCGGCAGTAAATGTTTGTGTCATG
TTTTAGCAATTCCTCATAGATGGAAGCTGCTGTCCTGGGGTTGGGTAGGGGT

A9018:GATCAAGGCTCCTGCACAGTGACTACATGAACATGACTCCTCGCCGACCAGG
GCCAACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAG

A9019:GATCCTGCGAAGTCGCGTGGTGGGGCATAGGGCTGGTAATGCTTGCGGGTT
GGCCCTGGTCGGCGAGGAGTCATGTTCATGTAGTCACTGTGCAGGAGCCTT

A9020:GATCAATGATCGAAACATACAACCAAACTTCTCCCCGATCTGCGGCCACTGGA
CTGCCCATCAGCATGAAAG

A9021:GATCCTTTCATGCTGATGGGCAGTCCAGTGGCCGCAGATCGGGGAGAAGTTT
GGTTGTATGTTTCGATCATT

D7001:
GATCACAGAACCAGCTCTATVVSVNSNTAAATCTAGGACGAAGAGAGGAGTACVVSV
NSNTAGACAAGAGACGTGGCCGGGACCCTGAGATGG

D7002:
GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCTANSNBSBBGTACTCCTCTCTTC
GTCCTAGATTTANSNBSBBATAGAGCTGGTTCTGT

FIG. 4(contd.)

FIG. 5

THE AMINO ACID SEQUENCE OF SEQUENCE BLOCKS

The single letter amino acid code is used

SB1	GSGQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGS
SB2	GSRKNPQEGL**YNEL**QKDKMAEA**YSEI**GMKGERGS
SB3	GSRGKGHDGL**YQGL**STATKDT**YDAL**HMQAGS
SB4	GSYEKSDGV**YTGL**STRNQET**YETL**KHEKPGS
SB4*	GSYEKSDGV**YTGL**STRNQET**YDTL**KHEKPGS
SB5	GSGNKVPEDRV**YEEL**NIYSAT**YSEL**EDPGEMSPGS
SB6	GSKQTLLPNDQL**YQPL**KDREDDQ**YSHL**QGNQLRGS
SB7	GSALLRNDQV**YQPL**RDRDDAQ**YSHL**GGNWARNKGS
SB8	GSQNKERPPPVPNPD**YEPI**RKGQRDL**YSGL**NQRRIGS
SB9	GSHVDNE**YSQP**PRNSRLSA**YPAL**EGVLHRSGS
SB10	GSPPRTCDDTVT**YSAL**HKRQVGD**YENV**IPDFPEDEGS
SB11	GSEYEDENL**YEGL**NLDDCSM**YEDI**SRGLQGTYQDVGS
SB12	GSKAGMEEDHT**YEGL**DIDQTAT**YEDI**VTLRTGEVGS
SB13	GSPLPNPRTAASI**YEEL**LKHDTNI**YCRM**DHKAEVAGS
SB28	GSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAGS
SB29	GSMIETYNQTSPRSAATGLPISMKGS

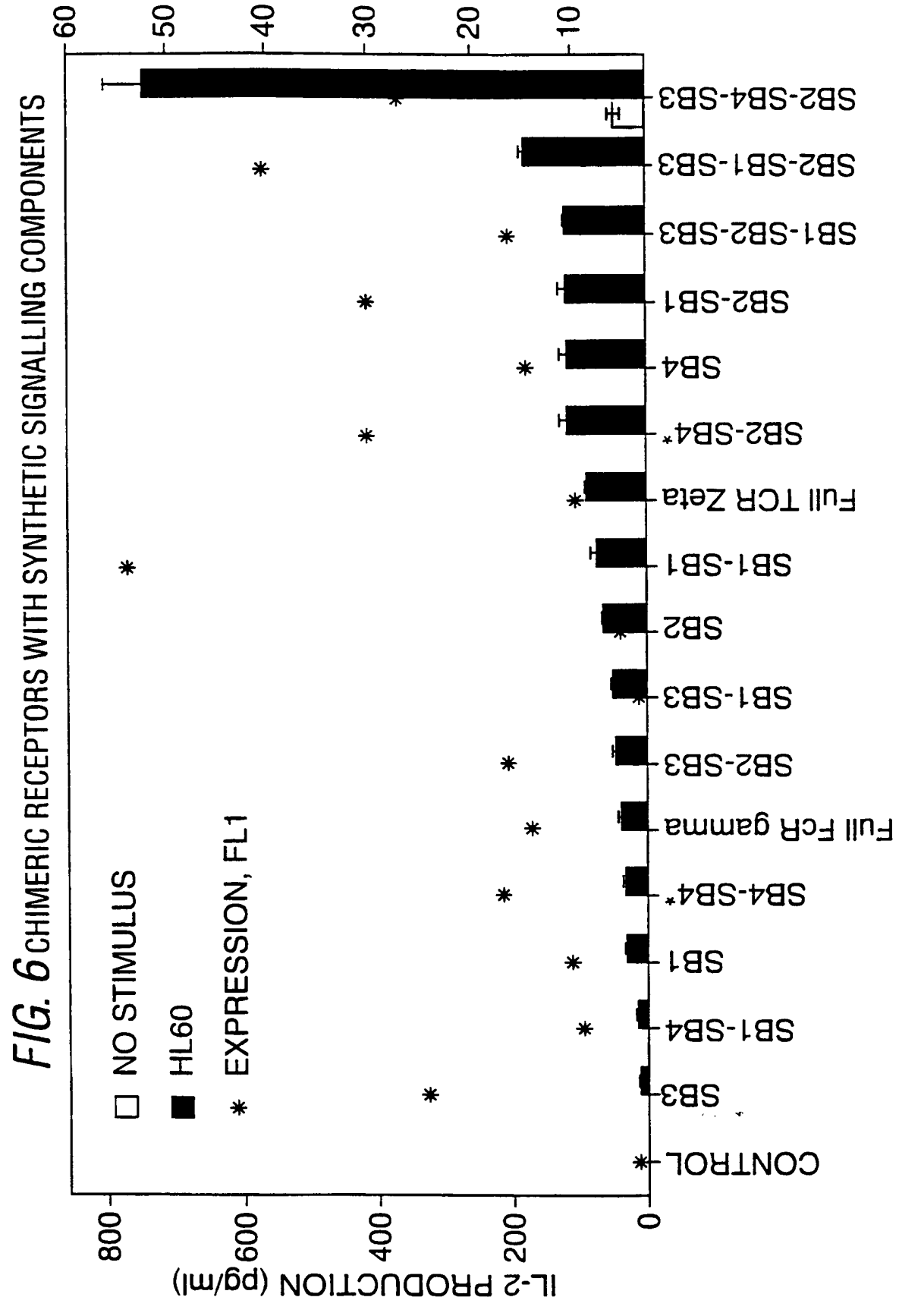


FIG. 6 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

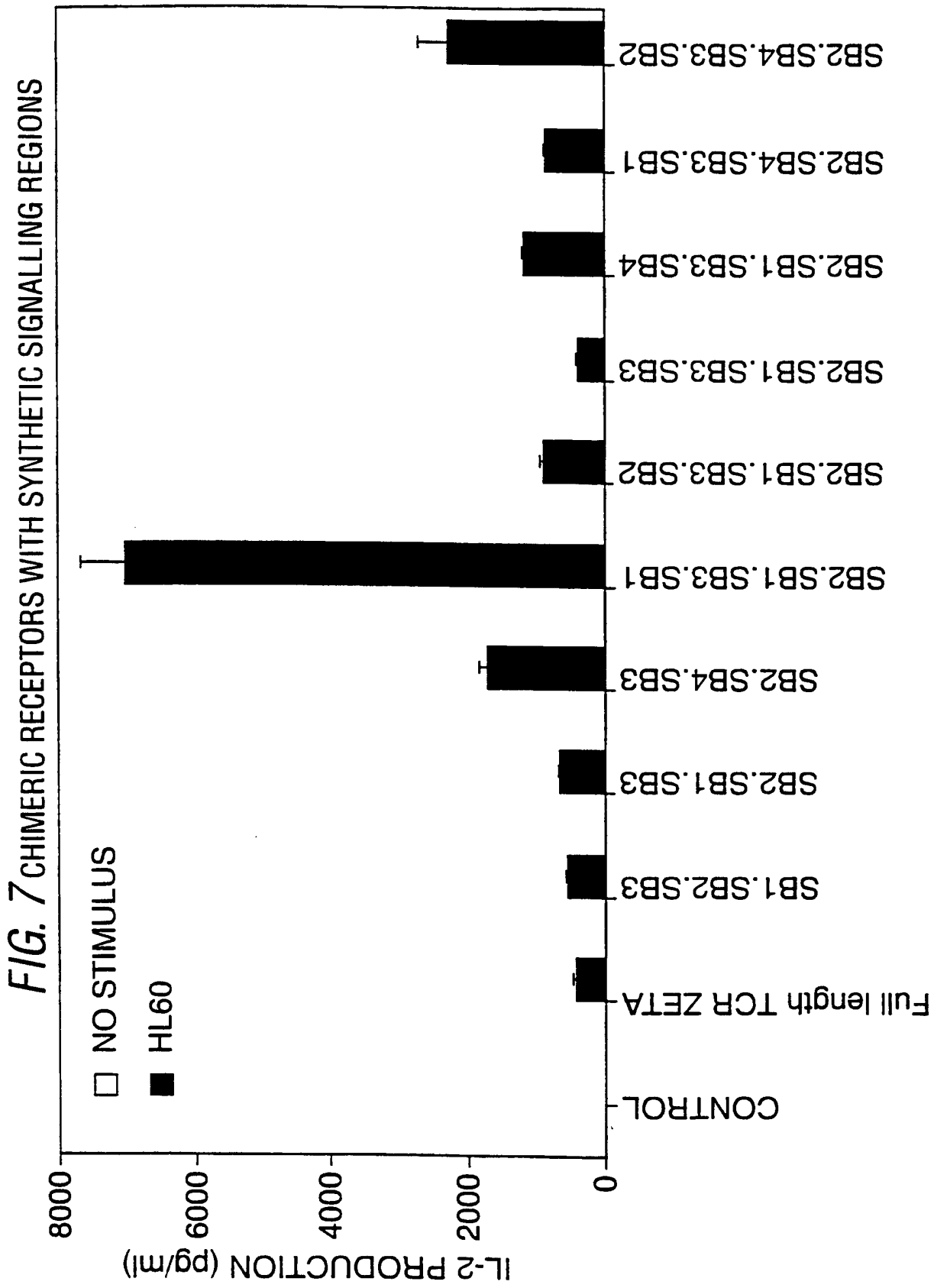


FIG. 7 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING REGIONS

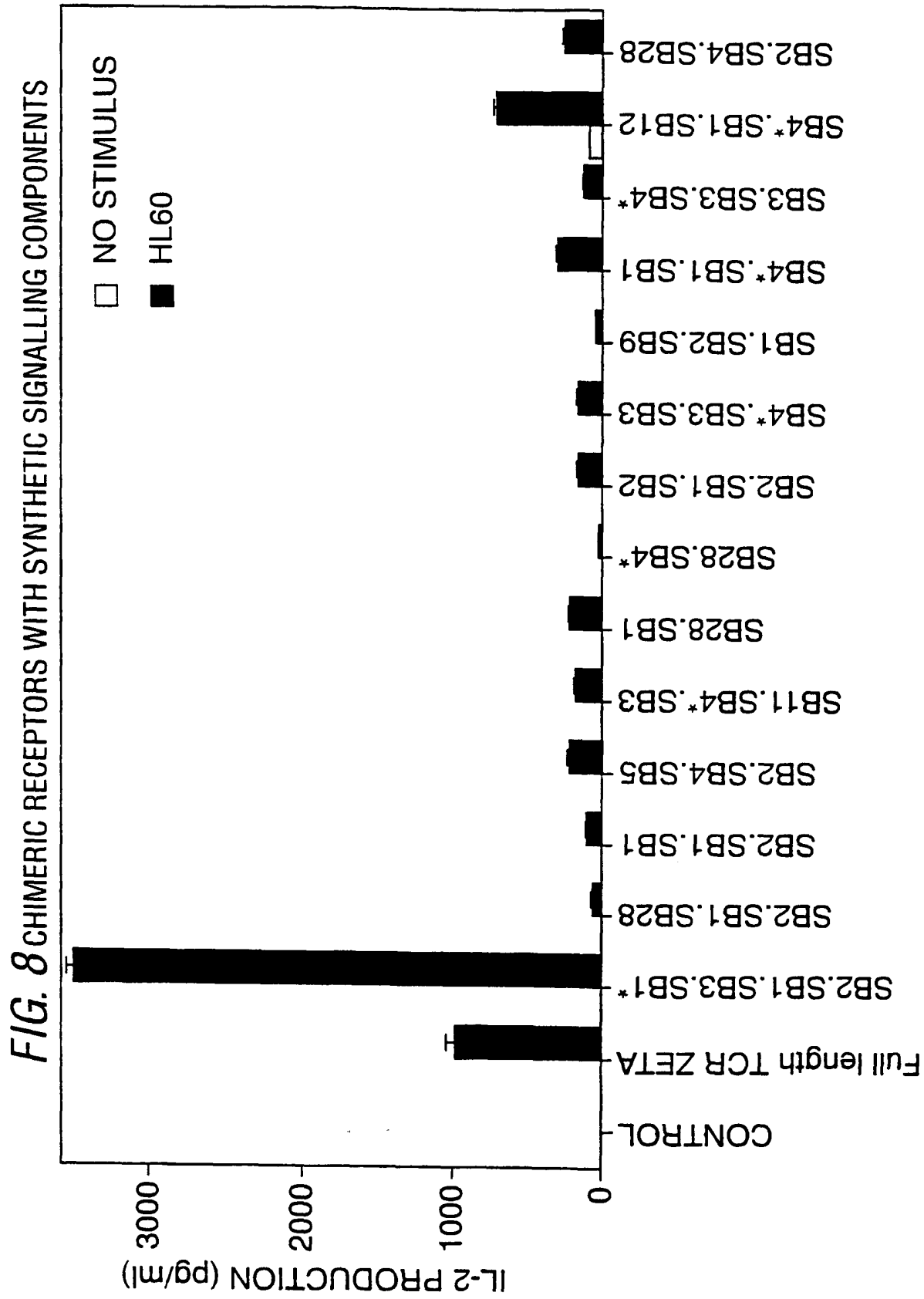


FIG. 8 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

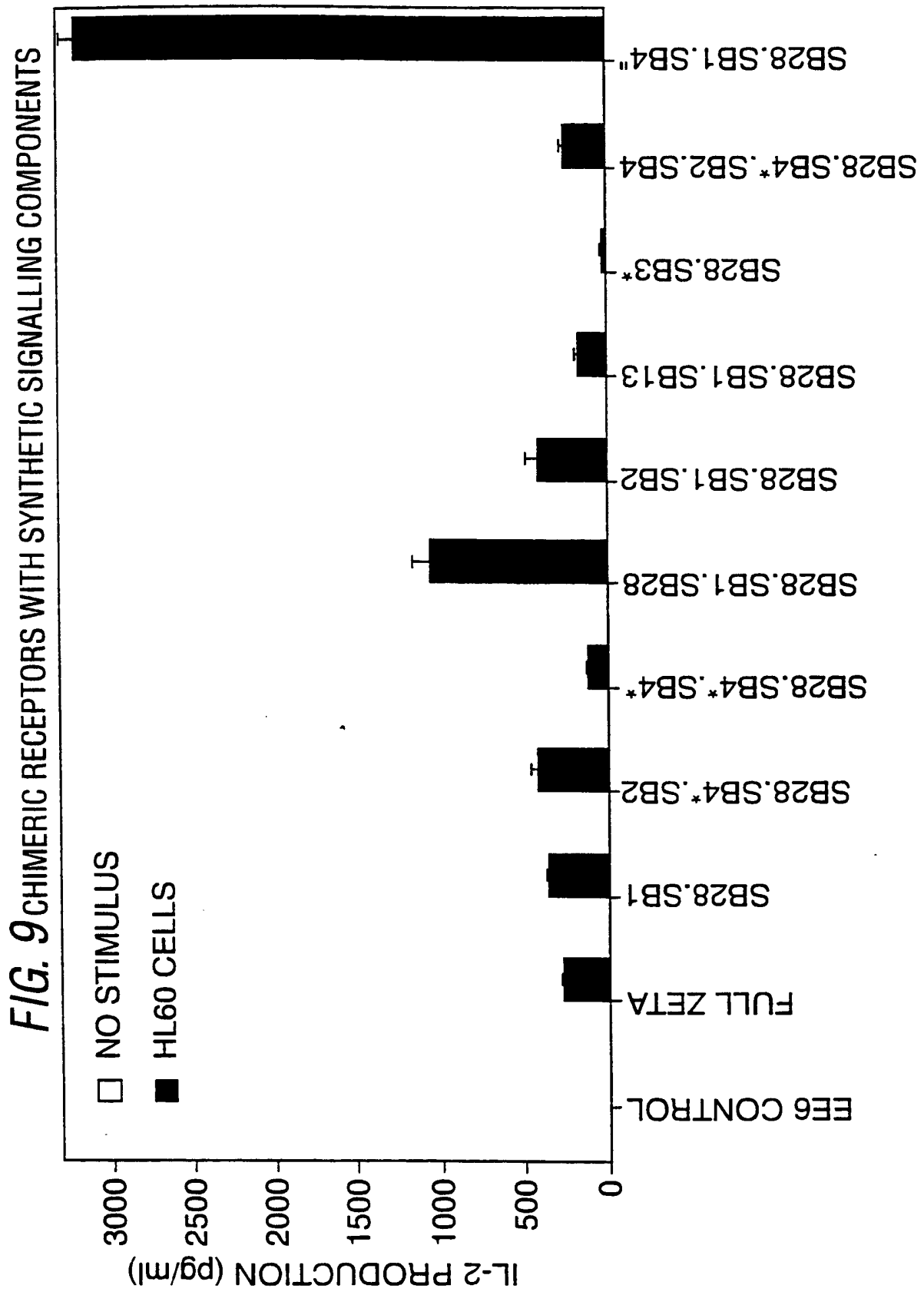


FIG. 9 CHIMERIC RECEPTORS WITH SYNTHETIC SIGNALLING COMPONENTS

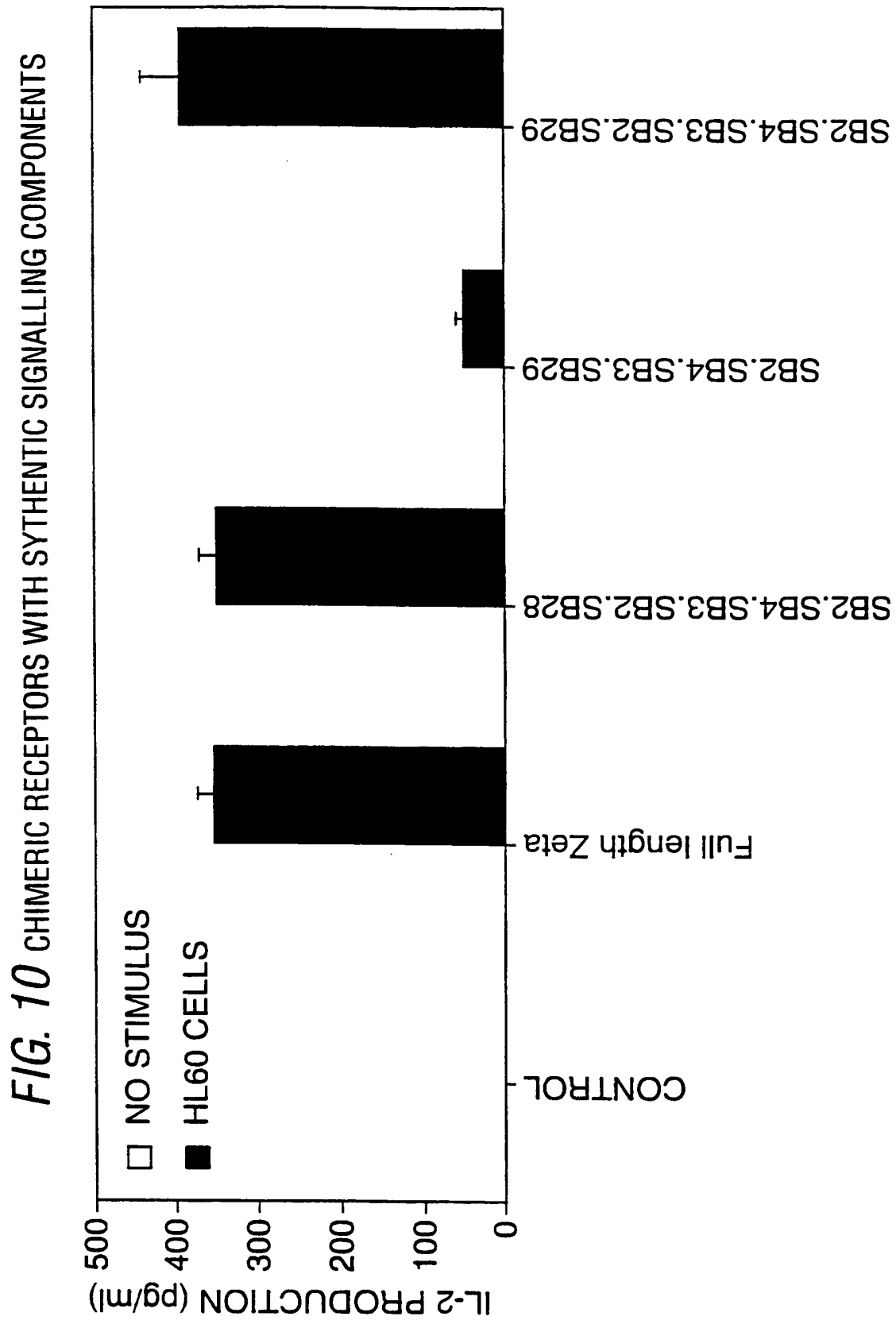


FIG. 10 CHIMERIC RECEPTORS WITH SYTHENTIC SIGNALLING COMPONENTS

INTERNATIONAL SEARCH REPORT

Int Application No
PCT/GB 00/01498

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12N15/12 C12N15/66 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, STRAND, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 17811 A (CHROMAXOME CORP) 30 April 1998 (1998-04-30) claims 1-26; figure 5E	3-10, 28-32
A	WO 90 00626 A (BAYLOR COLLEGE MEDICINE) 25 January 1990 (1990-01-25) the whole document	
A	DE 44 23 183 A (BIRSNER & GROB BIOTECH GMBH) 4 January 1996 (1996-01-04) the whole document	
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 August 2000	31/08/2000

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

In Application No
PCT/GB 00/01498

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